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TITLE: Selective Inhibition of T Cell Tolerance as a Means of
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Cancer

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13. ABSTRACT (Maximum 200 Words) Purpose: To determine if the addition of Go6976 to vaccine protocols will inhibit the re-induction of neu specific tolerance and thus facilitate immune mediated protection against breast cancer. Scope: In the Her-2/neu model of spontaneous breast cancer the immune system of these transgenic mice are tolerant to the neu protein. While immunity to neu can be demonstrated in the neu-transgenic mice (partial breaking of tolerance), this immunity is inadequate in terms of preventing the spontaneous development of tumors and preventing death from tumor challenge. Our experiments involve concomitantly treating mice with the PKC inhibitor Go6976 during tumor vaccine therapy in order to prevent tolerance induction and enhance immunotherapy. Findings: In initial experiments death from tumor was delayed by approximately 1 week in treated mice versus untreated controls. Likewise, in a second experiment non of the untreated mice (0/10) demonstrated evidence of neu-specific T cell responses, while 1/10 treated mice demonstrated neu-specific T cells. Importantly, the responder mouse had no evidence of tumor. Significance: These initial experiments while not dramatic suggest that the administration of Go6979 can impact immunotherapy. With these initial studies as a guide the major focus now is optimizing the delivery of the drug and vaccine.				
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Introduction:

In the *Her-2/neu* model of spontaneous breast cancer development it is clear that the immune system of these transgenic mice are tolerant to the neu protein (1-3). In this model not only does the overexpression of neu lead to tumorigenesis but the neu protein is the target of both humoral and cellular immunity which prevent tumor-induced death in the non-transgenic mice (1, 4). Indeed, while immunity to neu can be demonstrated in the neu-transgenic mice (partial breaking of tolerance), this immunity is inadequate in terms of preventing the spontaneous development of tumors and preventing death from tumor challenge. We have demonstrated *in vitro* that the PKC inhibitor Go6976 has the ability to selectively inhibit TCR induced tolerance induction while only minimally inhibiting T cell activation. We hypothesize that the addition of Go6976 to vaccine protocols will inhibit the reinduction of neu specific tolerance and thus facilitate immune mediated protection against the development of spontaneous breast cancer development and tumor challenge.

Body:

Initial studies were preformed in order to determine the optimal dosing and kinetics of the administration of Go6976 in relation to the tumor vaccine. In as much as this is a novel approach to tumor immunotherapy there was no precedent to guide us. Based on studies employing PKC inhibitors *in vivo* for other purposes we started with a dose of 5mg/kg q3 days for 3 doses (5). The mice were treated as follows:

NT2.5 Tumor
Challenge 10 n/n Mice (10⁵cells/mouse) $\xrightarrow{4 \text{ days}}$ 3T3 NeuGM Vac 3x10⁶cells/mouse $\xrightarrow{3 \text{ days}}$ DMSO or PKCi injection X 3
(~every 3 days)

Figure 1

We treated 10 mice in the DMSO group and 10 in the Go6976 (PKCi) group. At approximately day 30 both sets of mice had developed tumors with the exception of one mouse in the treated group. At this time, lymphocytes were harvested from the spleens of both sets of mice. The cells were restimulated *in vitro* and then assessed for activation by intracellular staining for IFN- γ . Only the mouse from the treated group displayed evidence of a neu-specific T cell response (Figure 1). Of note, none of the Go6976 treated mice displayed any adverse effects of the drug. While these data are not dramatic, we interpreted them as an indication

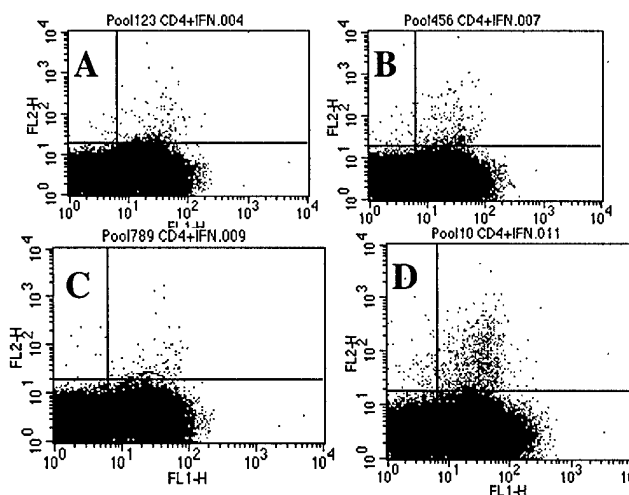


Figure 2: Spleens were harvested and incubated for one week with irradiated FVB/n splenocytes as APCs, and irradiated 3T3-neu cells as a source of antigen. The T cells were isolated and stimulated overnight with irradiated, IFN- γ treated 3T3-neu cells and then they were evaluated for intracellular IFN- γ production. The plots above are pooled T cells from the Go6976 treated mice in which tumors grew (A-C) and the treated mouse which rejected its tumor (D)

that the drug might have had a positive immunologic effect. More importantly, it provided a framework on which to build a more effective dosing regimen.

Based upon these initial experiments we doubled the dose of the drug to 10mg/Kg IP q3 days this time for 4 doses. As seen in Figure 2 beginning at about day 18 we saw a difference in the development of tumor between the untreated and Go6976 treated groups. By day 24 this difference was less pronounced. Note, even at this higher dose there was no evidence of toxicity. Once again we interpreted these data as indicating that the Go6976 was potentially delaying the induction of neu specific tolerance. The fact that the effect was lost indicated that we might need to maintain the PKCi treatment longer and perhaps decrease the dosing interval.

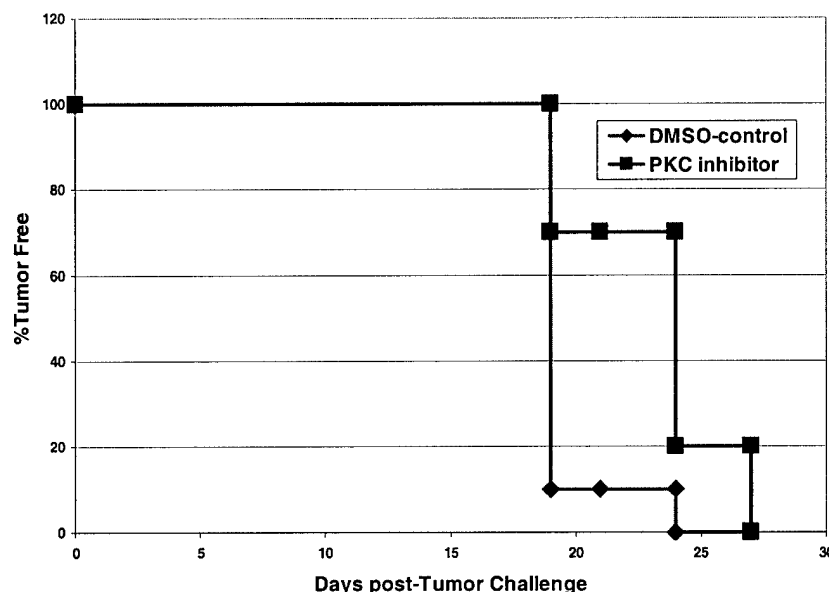


Figure 3: The mice were treated as described in Figure 1 with the exception that the dose was increased to 10mg/Kg IP q3 days for 4 doses.

Thus, our focus has been on trying to optimize the Go6976 dosing. However, during the course of our studies we noted that tumor growth in the vaccine treated mice (without Go6976 treatment) was more rapid than had been previously described (1, 4, 6). These observations forced us to examine each component of the model and delayed our efforts to maximize the dosing of the Go6976. This problem is illustrated in Figure 4 below. As seen in the figure the vaccine itself had no effect on decreasing tumor size as measured on day 20. Likewise, the addition of the drug had no impact. Thus it seemed as if the vaccine was not working as had previously been described.

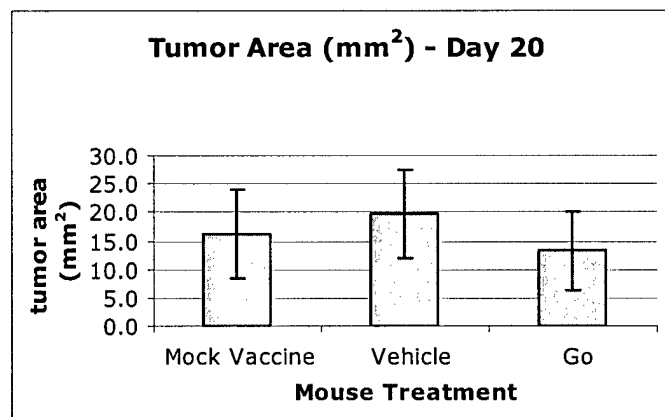


Figure 4: Mice were treated with no vaccine (Mock), Vaccine + vehicle or Vaccine + Go6976 (Go) as described for Figure 3. Tumor size was evaluated on day 20. Note all mice had evidence of tumor.

In order to try to resolve this problem we examined:

1. *The tumor:* We were concerned that tissue culture passage of the tumor had selected for a more virulent phenotype thus we thawed out new tumor. However, this did not solve the problem.
2. *Neu expression on the vaccine:* FACS analysis indicated that neu expression in the vaccine cells was sufficient (data not shown)
3. *GM-CSF secretion of the vaccine:* As shown below, the GM-CSF levels secreted by the vaccine were inadequate most likely accounting for the lack of efficacy. Thus we derived new vaccine cells and spontaneous secretion of GM-CSF levels were determined by ELISA.

	GM-CSF (pg/ml)
Old vaccine	3661
New vaccine	7417

Finally, new data has come to light in terms of the mechanism of tolerance in this model. It appears as if the Neu transgenic mice develop T regulatory cells against neu protein. Furthermore, it appears as if the ability of cytoxin to enhance the immune response in this model is due in part to its ability to eliminate such cells (6) and (E. Jaffee, unpublished findings). We had proposed the use of cytoxin in the original proposal as a potential condition to enhance the effect of Go6976 in suppressing tolerance. Given these new data we will proceed directly to integrating cytoxin into our regimen.

Key research Accomplishments:

- * Administration of Go6976 at escalating doses without toxicity.

- * Evidence of the ability of Go6976 to impact vaccine responses *in vivo*.
- * Re-optimization of vaccine protocol.

Reportable outcomes: None to date.

Conclusions:

We interpret our initial findings as suggesting that Go6976 can in fact have a positive impact on enhancing vaccine therapy. While these initial findings are not dramatic, they provide a framework from which we can develop the therapy in terms of maximizing the effectiveness of the drug and vaccine. Clearly, our studies have been hampered by the lack of efficacy of vaccine in general which we believe is due to suboptimal GM-CSF secretion. In this regard, an important aspect of this model is that the efficacy of immunotherapy is moderate at best. Thus, we believe we are testing the idea of tolerance suppression therapy under rigorous (perhaps more realistic conditions). Importantly, as we have escalated the dose, we have not observed any toxicity of the Go6976. We believe that in our next series of experiments, the introduction of cytoxan will enhance the likelihood that the Go6976 will be able to suppress neu-specific tolerance.

Optimizing the dosing schedule of the Go6976 still remains a major hurdle for us. In this regard, in another project in the lab we will be using sustained release devices that are inserted under the skin of the mice in order to deliver a steady rate of drug. If this technique looks promising then we can potentially integrate this system into these experiments.

Two major challenges of cancer vaccine therapy are to activate and expand the frequency of tumor specific T cells from a pool of already tolerized cells and to prevent tumor induced tolerance from prematurely terminating the efficacy of immunotherapy. In this model if we can demonstrate the ability of Go6976 to overcome these hurdles, then we can think about integrating such a strategy into current vaccine trials for breast cancer.

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